II. RESPONSE TO OFFICE ACTION

A. Status of the Claims

Claims 30-47 were pending and rejected in the Office Action mailed July 22, 2004 ("Action"). Claims 30, 31 and 41 are amended. Claims 48-49 have been added. Support for the amendments can be found in the Specification at least at page 99, line 30 to page 100, line 4 and in the originally filed claims. Therefore, no new matter has been added. Claims 30-49 are pending in this case.

B. Priority

Applicants erroneously disclaimed all priority in the previous response. With this RCE, Applicants claim the benefit to co-pending application Ser. No. 09/203,078 under 35 U.S.C. § 120.

C. IDS

Because Applicants are again claiming priority to Serial No. 09/203,078, they respectfully request that the SIDS filed on April 2, 2004 be reconsidered. A copy is provided. Also, a copy of Kraft *et al.* (C103) is included. Consideration of this reference is kindly requested.

Applicants also respectfully request that the Examiner kindly indicate on the record whether reference B8 listed in the IDS filed on August 22, 2001 was considered. In the initialed copy of that IDS that was sent to the Applicants with the Office Action mailed October 3, 2003, it is not clear whether this reference was considered. A copy of the initialed IDS is included for the Examiner's convenience.

D. Double Patenting Rejection

The Action rejects claims 30-43 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 70-104 of U.S. Patent 6,726,907 (the '907 patent) in view of U.S. Patent 5,553,309 (March). The earlier Office Action contends that the claims in the '907 patent differ from the instant claim by not reciting a total number of virus particles in the range of 5×10^{14} to 1×10^{18}. It argues that March teaches administering 10^{14} pfu of adenovirus to a host (col. 6, lines 50-52). It concludes that the stock of adenovirus was obvious. Applicants respectfully traverse this rejection.

Applicants assumed the rejection is referring to issued claims 1-35 in the '907 patent. As acknowledged by the Examiner, these issued claims do not recite a total number of virus particles as indicated in the present application. The present application relates to the invention of "very large quantities of adenovirus particles [that] can be produced using the processes of the present invention." Specification at page 4, lines 11-13. Applicants contend that the claims in the present application are patentably distinct for this reason.

March does not show how one could achieve an adenovirus composition of that scale. March does not concern production methods for adenovirus and provides no teaching regarding how one would achieve 10^{14} pfu of adenovirus. The claims of the '907 patent do not indicate this as well. Applicants point out that with a double patenting rejection, only the claims of the '907 application are used for the rejection. MPEP § 804. III. ("One significant difference is that a double patenting rejection must rely on a comparison with the claims in an issued patent or to be issued patent, whereas an obviousness rejection based on the same patent under 35 U.S.C. 102(e)/103(a) relies on a comparison with what is disclosed (whether or not claimed) in the issued or to be issued patent."). Consequently, there is no evidence of a reasonable expectation of success at achieving the claimed invention. No evidence is provided that the skilled artisan

would know or be able to achieve the claimed invention based on March or the claims of the '907 patent. For this reason, the claims in the present application are patentably distinct from the claims in the '907 patent. Applicants respectfully request this rejection be withdrawn.

Nonetheless, in the interests of expediting prosecution, Applicants are submitting a terminal disclaimer, which should render the rejection moot. Applicants note that submission of a terminal disclaimer is not an admission of nonobviousness.

E. Claims Are Not Anticipated by Inventors' PCT Application

The present application claims priority to Serial No. 09/203,078, which was filed on December 1, 1998. Therefore, the rejection of the claims over Zhang *et al.* (WO 00/32734), which is the PCT publication related to the priority document, is rendered moot.

F. Claims 30-40 Are Not Anticipated or Rendered Obvious by Condon *et al.*

The Action rejects claims 30-40 as anticipated or render obvious over Condon *et al.* (U.S. Patent No 6,168,944) ("Condon") because it states there are reasons to believe that the composition disclosed in Condon meets at least the particle/PFU element and the BSA element of the claims. Applicants respectfully traverse this rejection.

For a reference to anticipate a claimed invention, it must teach every element of the claimed invention. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987).

Claim 30 states, "A recombinant adenovirus composition comprising between 5×10^{14} and 1×10^{18} viral particles and having less than 50 ng of BSA per 1×10^{12} viral particles."

Condon does not teach a purified adenoviral composition with less than 50 ng of BSA per 1×10^{12} viral particles.

Contrary to the Action's contentions on page 5, the product disclosed by Condon does not reasonably appear the same as, or similar to, the claimed product. The Action relies on *In re Best*, 562 F.2d 1252, 1255, 195 U.S.P.Q. 430, 433-34 (CCPA 1977). However, it is not appropriate in the present case to place the burden on the patent owner to show that Condon does not teach the same product because the product of Condon was **not** "produced by identical or substantially identical processes" as compared to the claimed product. The Action cites the Condon patent as showing that cells were rapidly washed to remove serum in step (i) at col. 8, lines 1-15. However, Condon teaches several sentences later that "in step (vi) above the phrase 'rapidly neutralizing the trypsin by ***adding serum***' means adding the appropriate column of serum at a rate of 1-3 liters per minute, preferably about 2 liters per minute." Condon at col. 8, lines 23-27 (emphasis added). Thus, to the extent that the Action relies on step (i) to achieve the BSA limitation of the claim, the consequences of this step appear to be nullified by subsequent step (vi).

Therefore, the Action has not demonstrated on this record that Condon was produced by a comparable method. Consequently, it is inappropriate to invoke *In re Best* and shift the burden of proof to Applicants.

The other rejected claims (claims 31-40) are multiply dependent, directly or indirectly, on claims 30 or 41. Claim 41 has not been rejected and consequently, because claim 30 is not anticipated or rendered obvious by Condon, these other claims are not anticipated or obvious for the same reasons.

Moreover, claim 31 recites a composition "having between about 50 pg and 10 ng of contaminating human DNA per 1 x 10^{12} viral particles . . . ." Claim 41, which was not rejected,

also recites a composition "having between about 50 pg and 10 ng of contaminating human DNA per 1 x 10^{12} viral particles." Accordingly, the recited element in claim 31 is free of the prior art.

Elements of claims 30, 31 and 43 are not taught by Condon. Consequently, this reference can neither anticipate nor render obvious these claims. Moreover, the remaining claims are dependent from one of these claims and thus, they also are not anticipated or obvious over Condon. Applicants respectfully request this rejection be withdrawn.

G. Claim 43 Is Not Obvious over Condon in view of Shabram or Huyghe

The Action rejects claim 43 as obvious over Condon in view of Shabram (WO 96/27677) or Huyghe *et al* (C44) (""Huyghe paper") for the reasons specified in the Office Action mailed October 3, 2003 ("2003 Action"). While it acknowledges that Condon does not teach a highly purified virus preparation, it contends that Condon teaches a large scale adenovirus preparation. It alleges that purification of Condon's batch by the chromatography method of the Huyghe paper would result in a batch meeting the purity requirements of the claim. It further argues that the skilled artisan would have been motivated to combine the references with a reasonable expectation of success because both share the ultimate goal of producing recombinant virus suitable for gene therapy. Applicants respectfully traverse this rejection. No reason is provided why the combination of Condon and Shabram renders claim 43 obvious, and therefore, a proper *prima facie* case has not been made.

Claim 43 depends from either claim 30 or claim 41, which both are directed to a recombinant adenovirus composition having between 5 x 10^{14} and 1 x 10^{18} viral particles. Claim 30 additionally indicates the composition has "less than 50 ng of BSA per 1 x 10^{12} viral particles." Claim 41 also indicates the composition has "between about 50 pg and 10 ng of contaminating human DNA per 1 x 10^{12} viral particles."

As discussed in the previous section, Condon does not teach the limitations in claim 30 and claim 41 was not rejected over Condon. Therefore, all of the elements recited in claim 43 have not been met because this claim depends from either claim 30 or 41. A proper *prima facie* case of obviousness requires that the combination of references teach each element of the rejected claim. The Huyghe paper does not address this defect. In the 2003 Action, the Examiner indicated that claim 40, which was directed to "between about 5 ng and 40 ng BSA per 1 x 10^{12} viral particles," was not rejected over the Huyghe paper. Claim 30 has a similar limitation. Therefore, because the combination of references does not teach the elements of either claim 30 or claim 41, from which claim 43 depends, the elements of claim 43 are also not taught. Consequently, this claim is not obvious and Applicants respectfully request this rejection be withdrawn on this basis.

H. Claim 30, 31, 34, 36, and 43 Are Not Obvious over Either Huyghe Reference

The Action rejects claims 30, 31, 34, 36, and 43 under 35 U.S.C. § 103(a) over the Huyghe paper or Huyghe *et al.* (WO 96/27677). It contends that the reference teaches a highly purified adenovirus composition that has less than 1,000 to 10,000-fold less virus than the claimed invention. Applicants respectfully traverse this rejection.

Claim 40 was not rejected over the Huyghe references. A purity limitation consistent with the recitation of claim 40 is indicated in the present claims. Claim 40 recites "between about 5 ng and 40 ng BSA per 1 x 10^{12} viral particles." Rejected claim 30 indicates the composition has "less than 50 ng of BSA per 1 x 10^{12} viral particles," and thus, Applicants contend that claim 30 is patentable over the cited art.

Rejected claim 31 recites a composition having "between about 50 pg and 10 ng of contaminating human DNA per 1 x 10^{12} viral particles." There is no evidence that this purity limitation is taught by either of the cited Huyghe references. Consequently, a proper *prima facie*

case has not been made. Claim 41 now has a similar limitation as claim 31. Also, claim 41 was not earlier rejected. The remaining rejected claims 34, 36, and 43 are dependent from claims 30 or 41, both of whose elements are not taught, as discussed above. Consequently, the elements of claims 34, 36, and 43 are not taught as well.

Therefore, the present claims are patentable over the Huyghe references.

I. Claims 30-38 and 43 Are Not Obvious over Blanche *et al.*

The Action rejects claims 30-38 and 43 as obvious over Blanche *et al.* (WO 98/00524) ("Blanche") (*also* U.S. Patent 6,485,958). Applicants respectfully request this rejection.

Claim 39 was not rejected based on Blanche. Claim 39 recites a composition having "between about 1 ng and 50 ng of BSA per 1 x 10^{12} viral particles." Rejected claim 30 recites a composition having "less than 50 ng of BSA per 1 x 10^{12} viral particles." Therefore, claim 30 is similarly free of the prior art.

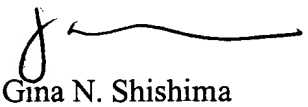
Rejected claims 31-38 and 43 depend either from claim 30 or from claim 41. Claim 41 has not been rejected. Because claim 30 is free from the prior art for the reason se forth in the previous paragraph, all of the pending claims should be free of Blanche. Applicants respectfully request this rejection be withdrawn.

CONCLUSION

Applicants believe that the foregoing remarks fully respond to all outstanding matters for this application. Applicants respectfully request that the rejections of all claims be withdrawn so they may pass to issuance.

Should the Examiner desire to sustain any of the rejections discussed in relation to this Response, the courtesy of a telephonic conference between the Examiner, the Examiner's supervisor, and the undersigned attorney at 512-536-3081 is respectfully requested.

Respectfully submitted,



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Date: November 22, 2004

Form PTO-1449 (modified)

List of Patents and Publications for Applicant's

INFORMATION DISCLOSURE STATEMENT

(Use several sheets if necessary)

Atty. Docket No.	Serial No.
INRP:081USD1/SLH	09/203,078
Applicants	
Shuyuan Zhang, Capucine Thwin, Zheng Wu, Toohyon Cho,and Shawn Gallagher	
Filing Date:	Group:
6/12/2001	Unknown

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 1*	*See Page 1*

U.S. Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Name	Class	Sub Class	Filing Date if App.
MM	A1	4,352,883	10/05/82	Lim			

Foreign Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Country	Class	Sub Class	Translation Yes/No
MM	B1	WO 94/17178	08/04/94	PCT			
	B2	WO 96/27677	09/12/96	PCT			
	B3	WO/98/00524	01/08/98	PCT			
	B4	WO 97/08298	03/06/97	PCT			
	B5	EP 0273085	12/29/86	Europe			
	B6	WO 98/22588	5/28/98	PCT			
	B7	WO 98/26048	6/18/98	PCT			
	B8	WO 98/54441	10/28/99	PCT			

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
MM	C1	Aboud *et al.*, "Rapid purification of extracellular and intracellular moloney murine leukemia virus," *Arch. Virol.*, 71:185-195, 1982.
	C2	Arap *et al.*, "Replacement of the *p16/CDKN2* gene suppresses human glioma cell growth," *Cancer Res.*, 55:1351-1354, 1995.
	C3	Baichwal and Sugden, *In:* Kucherlapati R, ed. Gene transfer. New York: Plenum Press, pp. 117-148, 1986.

25065727.1

Examiner: Mdm **Date Considered:** 9-30-03

EXAMINER: initial if reference considered, whether or not citation is in conformance with MPEP609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Shuyuan Zhang *et al.*

Serial No.: 09/880,609

Filed: June 12, 2001

For: AN IMPROVED METHOD FOR THE PRODUCTION AND PURIFICATION OF ADENOVIRAL VECTORS

Group Art Unit: 1648

Examiner: Mary Mosher

Atty. Dkt. No.: INRP:081USD1

CERTIFICATE OF MAILING
37 C.F.R 1.8

I certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to:, Attn.: Mary Mosher, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date below:

April 2, 2004 — Michael R. Krawzsenek
Date

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Attn.: Mary Mosher
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

In compliance with the duty of disclosure under 37 C.F.R. § 1.56, it is respectfully requested that this Information Disclosure Statement be entered and the documents listed on attached Form PTO-1449 be considered by the Examiner and made of record.

In accordance with 37 C.F.R §§ 1.97(g), (h), this Information Disclosure Statement is not to be construed as a representation that a search has been made, and is not to be construed to be an admission that the information cited is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

This application is a divisional application of Serial No. 09/203,078, filed December 1, 1998, and is relied upon for an earlier filing date under 35 U.S.C. § 120. In accordance with Rule 37 C.F.R. § 1.98(d) copies of the listed documents are not enclosed as they have been previously cited by or submitted to the Patent and Trademark Office in prior application Serial No. 09/203,078.

A fee as set forth in 37 C.F.R. § 1.17(p) in the amount of $180.00 is enclosed herewith. If an appropriate check has not been enclosed, or if it is insufficient, the Commissioner is authorized to deduct the appropriate fee from Fulbright & Jaworski Account No.: 50-1212/INRP:081USD1.

Applicants respectfully request that the listed documents be made of record in the present case.

Respectfully submitted,

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Date: April 2, 2004

Form PTO-1449 (modified)	Atty. Docket No. INRP:081USD1	Serial No. 09/880,609
List of Patents and Publications for Applicant's INFORMATION DISCLOSURE STATEMENT	Applicant Shuyuan Zhang *et al.*	
(Use several sheets if necessary)	Filing Date: June 12, 2001	Group: 1648

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 1*	*See Page 1*

U.S. Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Name	Class	Sub Class	Filing Date of App.
	A6	4,725,547	2/16/88	Sakamoto *et al.*	435	239	8/9/85
	A7	4,797,368	1/10/89	Carter *et al.*	435	320	3/15/85
	A8	5,139,941	8/18/92	Muzycka *et al.*	435	172.3	10/25/91
	A9	5,552,309	9/3/96	March	435	172.3	9/30/94
	A10	5,607,851	3/4/97	Pellegrini *et al.*	435	236	7/18/94
	A11	5,744,304	4/28/98	Munford	435	6	5/30/95
	A12	5,789,244	8/4/98	Heidrum *et al.*	435	320.1	1/8/96
	A13	5,837,520	11/17/98	Shabram *et al.*	435	239	3/07/95
	A14	6,194,210	2/27/01	Leu and Seifert	435	403	4/17/96

Foreign Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Country	Class	Sub Class	Translation Yes/No
	B9	1279843	11/10/89	Japan			ABSTRACT
	B10	4-9338	1/14/92	Japan			ABSTRACT
	B11	EP 0475623	3/18/92	Europe			
	B12	WO 00/34444	6/15/00	PCT			
	B13	WO 93/18790	9/30/93	PCT			
	B14	WO 93/25224	12/23/93	PCT			
	B15	WO 94/06910	3/31/94	PCT			
	B16	WO 95/10601	4/20/95	PCT			
	B17	WO 95/19427	7/20/95	PCT			
	B18	WO 95/25789	9/28/95	PCT			
	B19	WO 96/32116	10/17/96	PCT			

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EXAMINER: INITIAL IF REFERENCE CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

Form PTO-1449 (modified)	Atty. Docket No. INRP:081USD1	Serial No. 09/880,609
List of Patents and Publications for Applicant's	**Applicant** Shuyuan Zhang *et al.*	
INFORMATION DISCLOSURE STATEMENT		
(Use several sheets if necessary)	**Filing Date:** June 12, 2001	**Group:** 1648

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 1*	*See Page 1*

Foreign Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Country	Class	Sub Class	Translation Yes/No
	B20	WO 97/04803	2/13/97	PCT			
	B21	WO 98/35554	8/20/98	PCT			
	B22	WO 99/12568	3/18/99	PCT			
	B23	WO 99/41416	8/19/99	PCT			

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
	C104	Cartwright, "Fermenter design for animal cell cultures," Animal Cells as Bioreactors, Cambridge University Press, 58-63, 1994.
	C105	Carver *et al.*, "Transgenic livestock as bioreactors: stable expression of human alpha-1-antitrypsin by a flock of sheep," *Biotechnology NY*, 11:1263-1270, 1993.
	C106	Complaint Aventis Pharmaceuticals Products Inc. and Aventis Pharma, S.A., Plaintiffs, v. Introgen Therapeutics, Inc., Defendant. Civil Action No. 01-451 from the U.S. District Court for the District of Delaware, June 29, 2001.
	C107	Corveleyn and Remon, "Maltodextrins as lyoprotectants in the lyophilization of a model protein, LDH," *Pharm. Res.*, 13:146-150, 1996.
	C108	Croyle *et al.*, "Factors that influence stability of recombinant adenoviral preparations for human gene therapy," *Pharm. Dev. Technol.*, 3(3):373-383, 1998.
	C109	Fried and Bromberg, "Factors that affect the stability of protein-DNA complexes during gel electrophoresis," *Electrophoresis*, 18:6-11, 1997.
	C110	Hall *et al.*, "Stabilizing effect of sucrose against irreversible denaturation of rabbit muscle lactate dehydrogenase," *Biophys. Chem.*, 57:47-54, 1995.
	C111	Herman *et al.*, "The effect of bulking agent on the solid-state stability of freeze-dried methylprednisolone sodium succinate," *Pharm. Res.*, 11:1467-1473, 1994.
	C112	Kotani *et al.*, "Improved methods of retroviral vector transduction and production for gene therapy," *Human Gene Therapy*, 5:19-28, 1994

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Form PTO-1449 (modified)	Atty. Docket No. INRP:081USD1	Serial No. 09/880,609
List of Patents and Publications for Applicant's INFORMATION DISCLOSURE STATEMENT	Applicant Shuyuan Zhang *et al.*	
(Use several sheets if necessary)	Filing Date: June 12, 2001	Group: 1648

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 1*	*See Page 1*

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
	C113	Kotani, "Serum-free production of adenoviral vectors for gene therapy," Williamsburg BioProcessing Conference, Nov. 18-21, 1996.
	C114	Lentfer and Conde, "A rapid and inexpensive procedure for the purification of adenovirions," *Archives of Virology*, 56:189-193, 1978.
	C115	Lu *et al.*, "Coat protein interactions involved in tobacco mosaic tobamovirus cross-protection," *Virology*, 248:188-198, 1998.
	C116	Lueckel *et al.*, "Formulations of sugars with amino acids or mannitol--influence of concentration ratio on the properties of the freeze-concentrate and the lyophilizate," *Pharm. Dev. Technol.*, 3:326-336, 1998.
	C117	Montagnon, "Polio and rabies vaccines produced in continuous cell lines: a reality for vero cell line," *Develop. Biol. Standard.*, 70:27-47, 1989.
	C118	Mori *et al.*, "Frequent somatic mutation of the *MTS1/CDK4I* (Multiple Tumor suppressor/Cyclin dependent Kinase 4 Inhibitor) gene in esophageal squamous cell carcinoma," *Cancer Res.*, 54:3396-3397, 1994.
	C119	Nadeau *et al.*, *Biotechnology and Bioengineering*, 51:613-623, 1996.
	C120	Payment *et al.*, In: *Biotechnology Current Progress*, ed. P.N. Cheremisinoff *et al.*, Technomic Publishing, 1991.
	C121	Racher *et al.*, "Culture of 293 cells in different culture systems: cell growth and recombinant adenovirus production," *Biotechnology Techniques*, 9:169-174, 1995.
	C122	Sagrera *et al.*, "Study of the influence of salt concentration on Newcastle disease virus matrix protein aggregation," *Biochem. Mol. Biol. Int.*, 46:429-435, 1998.
	C123	Trepanier *et al.*, *Journal of Virological Methods*, 3:201-211, 1981.
	C124	Vanlandschoot *et al.*, "pH-dependent aggregation and secretion of soluble monomeric influenza hemagglutinin," *Arch. Virol.*, 143:227-239, 1998.
	C125	Vossen and Fried, "Sequestration stabilizes lac repressor-DNA complexes during gel electrophoresis," *Anal. Biochem.*, 245:85-92, 1997.
	C126	Wills and Menzel, "Adenovirus Vectors for Gene Therapy of Cancer," *J. of Cellular Biochem.*, Suppl: 17E, S216:206, 1993.

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Archives of Virology 57, 243—254 (1978)

Archives of Virology

Cell Cycle-Dependent Multiplication of Avian Adenoviruses in Chicken Embryo Fibroblasts[1]

By

V. Kraft and Ilse Tischer

Institute of Poultry Diseases, Free University Berlin, and
Robert-Koch-Institute, Department of Virology, Berlin

With 7 Figures

Accepted December 27, 1977

Summary

Propagation of CELO virus employing confluent monolayers of chicken embryo fibroblasts (CEF) yielded virus titers one to two logs lower than those from confluent chicken kidney (CK) cells. An enhancement of virus production in CEF as measured by plaque formation was obtained by infecting cultures in the growing non confluent state. Measurements of ^{3}H-thymidine incorporation revealed a positive correlation between the DNA synthesis of CEF cultures at the time of inoculation and the amount of progeny virus, whereas in the CK-CELO-system no such relation was observed.

Requirement of replicative fibroblasts for CELO multiplication was also demonstrated by comparison of virus replication in synchronized stationary and serum stimulated CEF cells. In stationary CEF cells arrested in the G_1 phase of the cell replication cycle by serum deprivation and infected with CELO virus, no cytopathic effect could be observed, and only very low amounts of virus were produced. But 24 hours after release of these cells for growth by serum stimulation a logarithmic rate of virus multiplication and a complete CPE occurred. Infection of synchronized CEF cultures at different stages of the cell cycle revealed that CELO multiplication was correlated with the S phase of the infected cell.

In synchronized CELO infected CEF cultures viral DNA synthesis started 12 to 14 hours after growth stimulation when cells were near the end of the S phase. In contrast, no viral DNA synthesis could be measured in growth arrested CELO infected CEF cells, when cellular DNA synthesis was low. Therefore not only production of infectious virus but also viral DNA synthesis is correlated with events during the S phase of the infected CEF cell.

[1] Presented in part at the Arbeitstagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Mainz, September 27—29, 1976.

0304-8608/78/0057/0243/$ 02.40

Introduction

Cell cycle-dependent multiplication has been described for some parvo- (15—18), papova- (14, 21, 22), picorna- (3, 19), a herpes- (9), and oncornaviruses (4—7, 20). While multiplication of these cited DNA- and picornaviruses is correlated with the S phase of the infected cell, oncornaviruses require mitosis of the infected cells for virus production (4—7).

CELO virus replicates in avian kidney and liver cells equally well, but infection of chick embryo fibroblasts yielded virus titers one to two logs lower than those from kidney and liver cells (2, 11, 12). The present studies were performed to elucidate the reason for the low multiplication rate in chick fibroblasts. It is shown that the amount of progeny virus depends on the DNA synthesizing activity of the infected CEF cultures. By use of synchronized chicken embryo cells multiplication of the avian adenovirus and synthesis of viral DNA was found to be dependent on events during S phase.

Materials and Methods

Viruses

The investigations were done with our own isolate of avian adenovirus, designated CELO Dahlem. Cross neutralization tests between the Phelps strain of CELO and our isolate indicated that these two strains were serologically indistinguishable. The CELO Dahlem virus was cloned and plaque purified, and repeated electronmicroscopic investigations gave no evidence of contamination by other viruses. Some comparable tests were done with 8 serologically distinct fowl adenoviruses (FA) which were kindly supplied by Dr. J. B. McFerran, Belfast, Northern Ireland (12).

Cells, Infectivity Assays, and Synchronization

CELO virus was grown in primary cultures of chicken embryo fibroblasts (CEF) and chicken kidney cells (CK). Fertile SPF chicken eggs (Valo) were obtained from Lohmann & Co., Cuxhaven, Federal Republic of Germany. Kidneys of fourteen day old SPF chickens grown in isolators were used for preparation of the CK cultures. Cells were grown at 38° C in a humidified CO_2 atmosphere in medium 199 with Hanks' salts (Flow) containing 10 per cent tryptose phosphate broth (TPB) and 1.2—6 per cent calf serum.

For plaque titrations in CEF 0.2 ml of serial tenfold virus dilutions were inoculated in 60 mm plastic petri dishes (Falcon Plastics), which were washed and overlaid with 5 ml of an Earle-lactalbumin (0.625 per cent)-agar-(Difco 0.75 per cent)-overlay containing 10 per cent calf serum after a one hour adsorption period. 3 ml of a second and third overlay were added on the second and fifth day of incubation, respectively. Plaques were counted after formalin-fixation (5 per cent) without staining on the ninth day. Plaque titrations in CK cells were done similarly but with only one agar-overlay and formalin-fixation on the fourth day after infection.

Cells were synchronized using a method similar to that described by HUMPHRIES and TEMIN (5, 6) by depletion of multiplication-stimulating activity from serum-containing medium. Tertiary or later cultures of CEF were plated in medium I containing 6 per cent calf serum at 6×10^5 cells per 60 mm dish. Four hours after plating, the medium was replaced by 5 ml of medium II with 1.2 per cent calf serum. Four days after changing to medium II 2.5 ml of medium III without calf serum were added.

^{3}H-Thymidine Incorporation

For measurement of the rates of ^{3}H-thymidine incorporation 10 µCi ^{3}H-thymidine (specific activity 26.7 Ci/mmol, purchased from Amersham Buchler, Braunschweig) in 0.1 ml medium without calf serum were added to each 60 mm dish with 5 ml medium. After incubation for 1 hour at 38° C cells were harvested by trypsinization and low speed centrifugation. Sedimented cells were lysed with 0.1 ml of an aqueous 5 per cent sodium dodecyl sulfate solution. Radioactivity of trichloroacetic acid-precipitable material was determined in a liquid scintillation counter (Nuclear-Chicago, Mark II).

DNA Labeling and Assays

Pulse-labeling of DNA, isolation and separation by isopycnic centrifugation in CsCl were performed as described by LUDWIG and ROTT (10).

Results

Comparison of the Multiplication of CELO Virus in CK- and CEF-Cultures

The differences in the multiplication of CELO virus in confluent CK- and CEF cells are illustrated by one step growth curves determined for these two cell systems (Fig. 1). Multiplication in CEF cultures was followed up to 144 hours p.i. because no complete CPE was detectable in these cultures. The slight decline of these curves towards the end of the experiment may be explained by thermal inactivation of the virus which is shown (Fig. 1, No. 5) for a virus suspension stored at 38° C for an equal time.

CELO virus multiplication in CEF cells shows a delayed and less progressive logarithmic virus growth curve, a shortened eclipse period, and titers are lower as compared with the CK system.

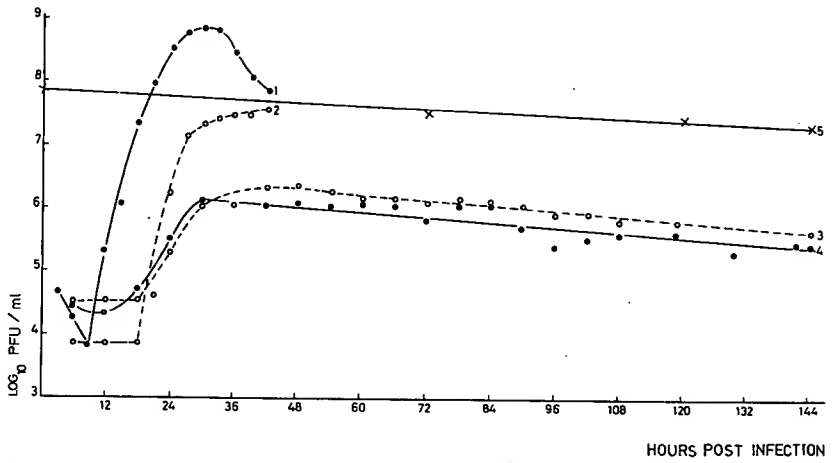

Fig. 1. One step growth curves of CELO virus in CK cells (1. cell-associated virus, 2. released virus) and CEF cultures (4. cell-associated, 3. released virus). Monolayers were infected at a multiplicity of about 70 PFU/cell. After 60 minutes at 4° C the inoculum was removed, the monolayers were washed twice with prewarmed PBS, and 5 ml of growth medium were added. Two plates were taken at indicated times. The cells were washed, overlaid with 5 ml growth medium and frozen. After three cycles of freezing and thawing, cell-associated and released virus was titrated in CK cells. No. 5: Inactivation of CELO virus infectivity on storage in growth medium at 38° C, titrated in CK cells

Factors Influencing Plaque Formation by CELO Virus in Chicken Embryo Fibroblasts

A decrease in the plaque number was obtained when secondary and tertiary CEF cultures were used for CELO virus titration instead of primary monolayers. Measurement of ^{3}H-thymidine incorporation by the cultures at the time of inoculation demonstrated that the decreasing sensitivity of secondary and tertiary CEF cultures for plaque formation by CELO virus was accompanied by a decrease in their DNA synthesis (data not shown).

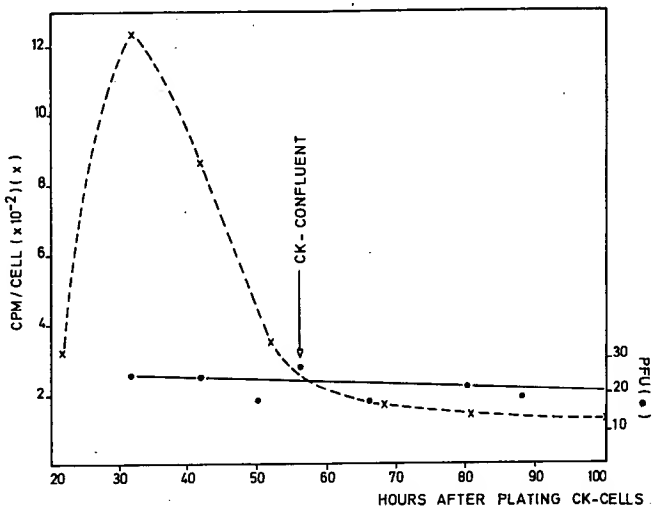

Fig. 2. Relationship between the number of plaques in CK cultures (•———•) infected with a standard dose of CELO virus at different times after plating, and the amount of ^{3}H-thymidine incorporation (× — — — ×) at the time of inoculation. Simultaneous to virus adsorption the rate of DNA synthesis was measured in parallel cultures

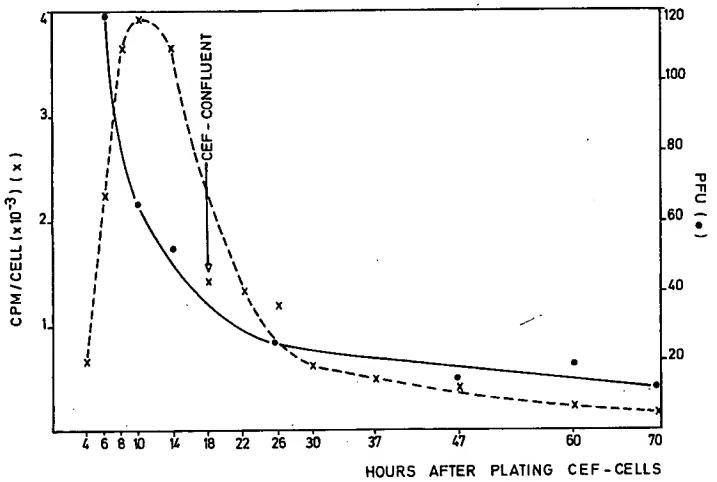

Fig. 3. Correlation between the number of plaques in CEF (•———•) infected with the same CELO virus doses at different times after plating of tertiary cells, and the rate of DNA synthesis (× — — — ×) at the time of inoculation

A dependence of virus multiplication on cell replication in CEF cultures became evident after the following experiments: Chicken kidney cells and chicken embryo fibroblasts were inoculated with a standard dose of CELO virus at different times after plating. In all these experiments CEF cultures were incubated for 216 hours, CK cultures for 92 hours. As shown in Figure 2, the degree of confluency and the varying DNA synthesizing activities of the CK cultures at the different times of inoculation did not influence the resulting virus titers. In contrast, a 6.5 fold enhancement of the plaque number was obtained when CEF cultures with high DNA synthesizing activity were inoculated as early as 8 hours after plating when they showed semiconfluency, as compared to cultures inoculated in the confluent state. When infection of CEF cultures was done later than 8 hours after plating the number of plaques decreased with increasing cell density. A concomitant decline in the cell DNA synthesis was measured in parallel cultures.

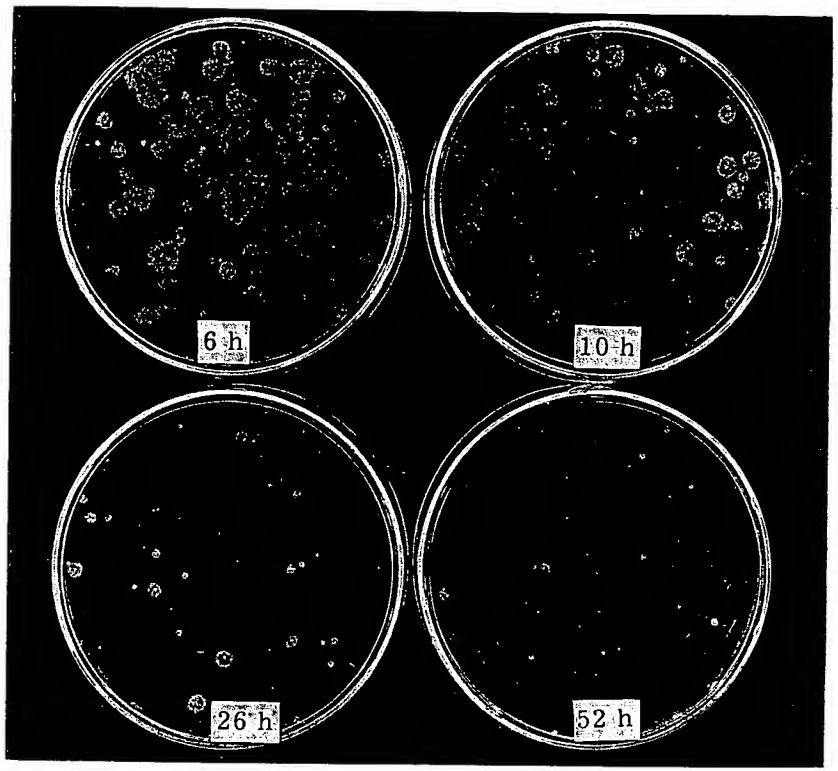

Fig. 4. Varying numbers and sizes of plaques in CEF infected with CELO virus at different times after plating. All cultures were infected with equal virus doses at the given times after plating

With CEF not only the number of plaques but also their size differed with varying degrees of confluency of the cultures at the time of inoculation. In cultures inoculated early after plating when cell DNA synthesis was high large plaques developed, whereas only small plaques were formed when confluent cultures with low DNA synthesis activity were inoculated with the same virus dose (Fig. 4).

Multiplication of Different Serotypes of Avian Adenovirus in CEF and CK Cells

To determine whether the results obtained with our CELO Dahlem strain would also hold for other fowl adenovirus serotypes multiplication in CEF and CK cultures of 7 other serologically distinct strains was investigated. For this purpose virus multiplication in tertiary CEF cultures inoculated 3 and 24 hours after plating was compared. Furthermore virus stocks were titrated in CEF and CK cultures.

As shown in Table 1, more than twice the number of plaques were formed when cultures were infected 3 hours after plating as compared to cultures infected in the confluent state 24 hours after plating. As already shown with the CELO Dahlem virus, titers of virus stocks of these other fowl adenoserotypes were in confluent CEF cultures 1.5 logs lower than titers in confluent CK cultures.

Table 1. *Multiplication of different serotypes of fowl adenovirus in CEF and CK cells*

Fowl Adeno—(FA) serotypes	PFU in tertiary CEF infected		Results of titrations in	
	3 hours after plating	24 hours after plating	CK	CEF
FA-1 (OTE)	n.d.[a]	n.d.	7.8[b]	6.4
FA-2 (SR-48)	76[c]	33	7.6	5.3
FA-3 (SR-49)	40	13	6.7	5.5
FA-5 (340)	25	14	7.2	5.2
FA-6 (CR-119)	171	67	7.1	6.0
FA-7 (YR-36)	250	125	7.2	5.3
FA-8 (Hungarian VI)	27	13	5.7	4.3

[a] n.d. = not done
[b] $\log_{10}$ PFU/0.2 ml
[c] Mean PFU of 5 cultures

Absence of Logarithmic Virus Multiplication in Synchronized and CELO Infected Stationary Chicken Embryo Fibroblasts

Multiplication of CELO virus in synchronized CEF cultures was also investigated by determining the number of infectious virus formed in stationary and growth released cultures at different times after infection. The effect of serum stimulation on cell growth is shown in Figure 5A: it led to a 2.5 fold increase of the cell number, whereas in the serum free cultures the number of cells remained constant throughout the experiment. As shown in Figure 5B, the onset of logarithmic virus multiplication could be induced at any given time if stationary cultures were released. Virus production in the stationary cultures was minimal even 120 hours p.i.

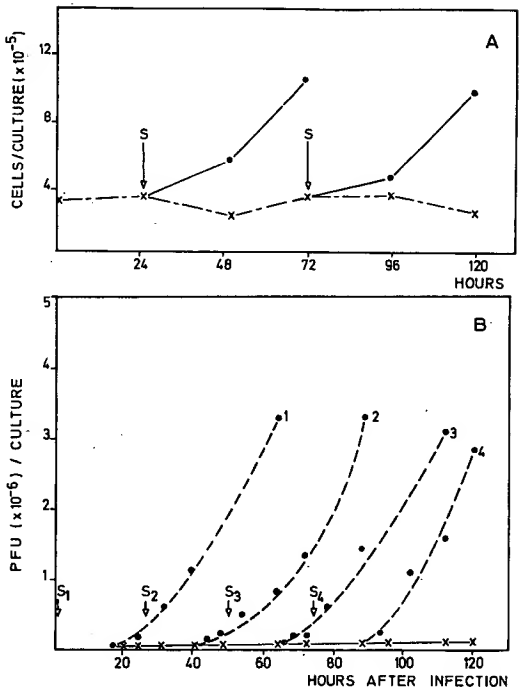

Fig. 5

A. Cell number of stationary CEF (× — — — ×), and in cultures stimulated by serum (S) (•———•) at the indicated times

B. Comparison of CELO multiplication in synchronized stationary (×———×) and serum stimulated (• — — — •) CEF. Stationary CEF, 3×10^5 cells per 60 mm dish, were exposed to CELO at a multiplicity of about 100 PFU per cell. Four groups of cultures were stimulated by addition of serum-containing medium at the indicated (S_1—S_4) times after infection. Total virus yield was determined by the plaque assay using CK cultures

S Phase Correlated Multiplication of CELO in Synchronized CEF Cultures

In order to determine the essential phase of the cell replication cycle for CELO virus multiplication CEF cells were synchronized by serum depletion and inoculated at different times after growth release. Total infectivity at different times p.i. was determined by plaque titration in CK cells (Fig. 6B). ^{3}H-thymidine incorporation of the cells was measured in parallel uninfected cultures (Fig. 6A). Whether cells were infected at the time of release (0), or 4, 8, and 12 hours, respectively, after growth release, the first appearance of infectious virus invariably coincided with the late S phase at about 12 to 16 hours after serum stimulation. Depending on the time of inoculation, the eclipse period varied from 15 hours (infection at time of growth release) to 8 respectively 7 hours (infection 4 respec-

tively 8 hours after growth release). When infection took place 12 hours after growth release at the beginning of late S phase the first appearance of newly formed virus coincided with the S phase of the following cell replication cycle (about 30 hours after release).

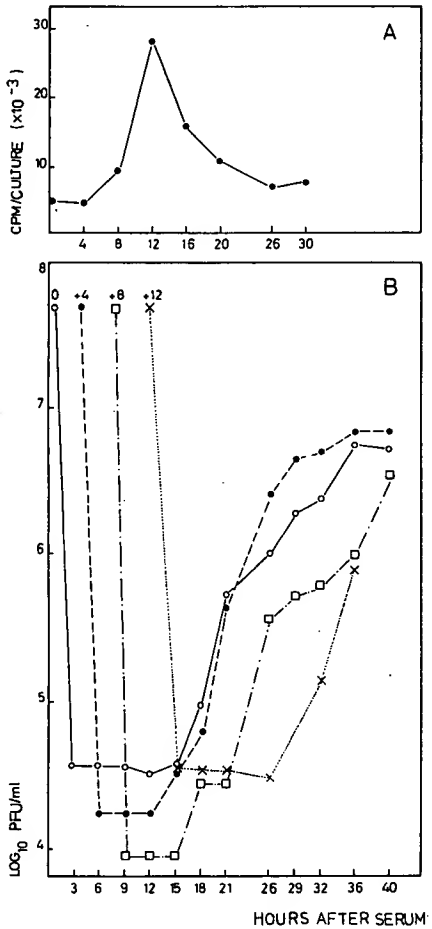

Fig. 6. Growth curves of CELO virus in synchronized CEF

A. Rate of DNA synthesis in parallel uninfected cultures at different points after addition of serum

B. Stationary CEF were released from growth inhibition by the addition of medium with serum. Infection was done with 100 PFU per cell at the time of release (o), and 4 (•), 8 (□), and 12 (×) hours after release. After 60 minutes adsorption of virus at 38° C the plates were washed twice. The points indicate total CELO yield, determined after three cycles of freezing and thawing by titration on CK cultures

Synthesis of Cellular and Viral DNA in Synchronized CEF Cells Infected With CELO

The necessity of cell replication for virus production could be confirmed by demonstrating coincidence of cellular and viral DNA synthesis in synchronized CEF cultures (Fig. 7, left side). At different times after infection and subsequent

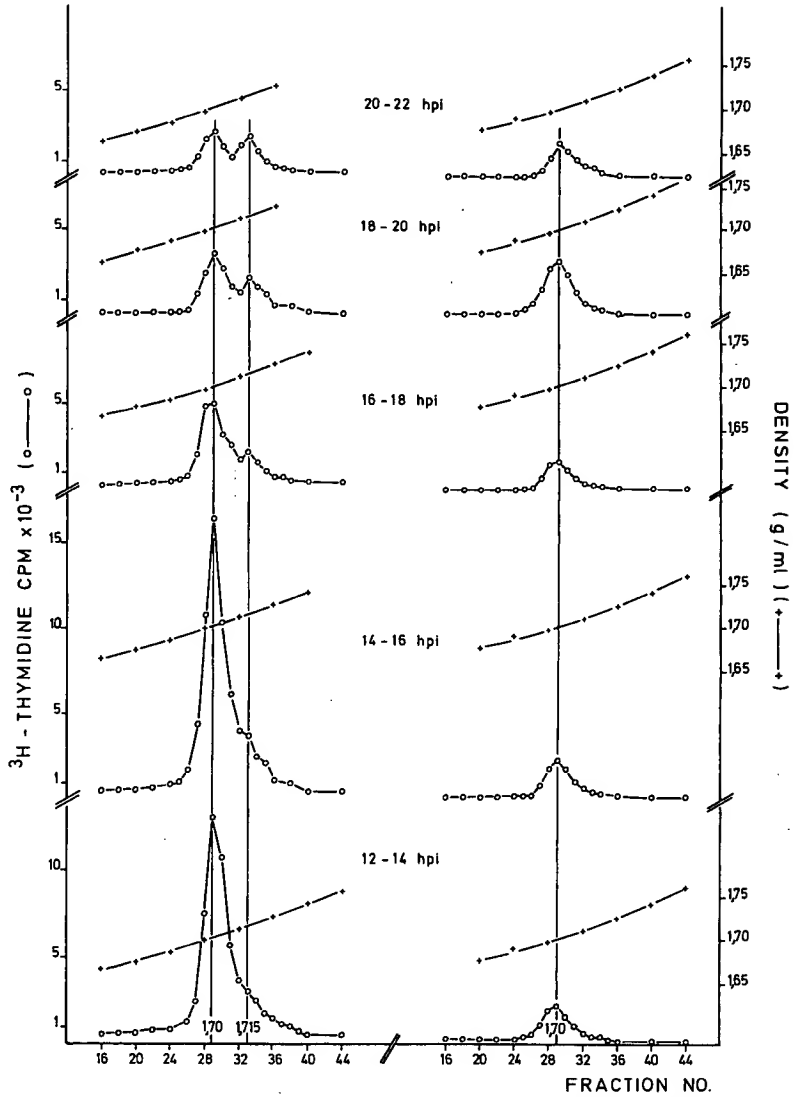

Fig. 7. CsCl-equilibrium density gradient analysis of ^{3}H-thymidine-labeled DNA from CELO infected synchronized CEF cells. DNA synthesized during the indicated 2 hour labeling periods in growth stimulated cells (left side), and by cells kept stationary in G_1 (right side)

growth stimulation, the cultures were pulse-labeled with ^{3}H-thymidine for 2 hours. The cellular and viral DNA were extracted and separated by isopycnic CsCl-centrifugation (10). The buoyant densities of cellular (1.70 g/cm^3) and viral DNA (1.715 g/cm^3) in CsCl had been determined using ^{14}C-labeled Adeno 2 DNA as a marker which was kindly provided by Dr. Doerfler (data not shown). As demonstrated in Figure 7 (left side), 12—14 hours p.i. additional to a peak of cellular DNA a small shoulder of viral DNA at the density of 1,715 g/cm^3 became visible, which increased at 14—16 hours p.i. After passing through the S phase peak of the cell cycle which appeared in this experiment 14—16 hours after growth stimulation of cells, cellular DNA synthesis decreased and viral DNA synthesis remained nearly constant from 16 to 22 hours p.i. In parallel tests with infected cultures kept arrested in G_1 phase only small peaks indicating a low amount of cellular DNA synthesis were found. Viral DNA could not be separated (Fig. 7, right side).

Discussion

As already described by MANCINI and YATES (11) and CHOMIAK *et al.* (2), CELO virus multiplies only poorly in chicken embryo fibroblasts as compared to chicken kidney cells. Experiments with a CELO virus strain isolated in our laboratory confirmed these observations.

As an explanation for this phenomenon MANCINI and YATES (11) discussed the heterogeneous source of fibroblastic cells from the whole decapitated embryo and the possibility that only one cell type might be permissive for CELO virus multiplication. To exclude a possible influence of epithelial cells present in primary cultures all tests were performed with tertiary or later cultures which are characterized by a highly homogeneous fibroblastic cell type.

However, CELO virus multiplication was correlated not only to the type of cell but also to its DNA synthesizing activity. This explanation was confirmed by the results obtained after infection of CEF at different stages of DNA synthesizing activity. Inoculation of growing cultures with a high rate of ^{3}H-thymidine incorporation led to 6.5 fold enhancement in the number of plaques with increased diameters as compared with confluent monolayers with a low DNA synthesis. Plaque counts decreased with increasing cell density at the time of inoculation which was accompanied by a decline in the rate of ^{3}H-thymidine incorporation of the cultures. The high permissiveness of CK cell cultures was found to be independent of their DNA synthesizing activity.

Seven other serotypes showed equal behaviour in their multiplication in CEF and CK cells like the fowl adenovirus of type FA-1 (CELO Dahlem) which was used for intensive studies. Therefore it can be concluded that the results found here may be of principle validity for avian adenoviruses.

The dependence of CELO virus multiplication on the replicative state of chicken embryo fibroblasts which is described here first for an adenovirus was further confirmed by experiments using synchronized fibroblasts. Methods as developed by HUMPHRIES and TEMIN (5, 6) for similar investigations on Rous sarcoma virus replication were used.

In infected CEF cultures which were kept stationary in the G_1 phase by depletion of multiplication-stimulating activity from serum-containing medium

even after 144 hours p.i. only minimal CELO virus production was demonstrable. In this experiment the logarithmic rate of virus multiplication could be provoked by addition of serum at any given time.

To answer the question whether a certain phase of the cell replication cycle might be essential for CELO virus multiplication, synchronized fibroblasts were infected at different stages of the cell cycle. The shortest eclipse period of 7 hours was observed when cultures were infected at the beginning of the S phase, whereas an eclipse period of 14—15 hours was found when the cultures were inoculated shortly after addition of serum to resting G_1 phase cells or at the end of the S phase.

A correlation between cellular DNA synthesis and viral DNA production became also evident in analysing the temporal course of viral and cellular DNA synthesis in infected and serum stimulated synchronized CEF cultures. On the other hand, in infected CEF cultures kept stationary in the G_1 phase, only low cellular DNA synthesizing activity and no viral DNA synthesis was detectable.

The observations presented here confirm the results of other authors who found that chicken kidney cells were permissive for CELO virus multiplication (1, 8, 13). This process, which leads to a cell lysis was found to be independent of the replicative state of the cultures. Multiplication of CELO virus in chicken embryo fibroblasts, however, is correlated to conditions of the cell connected with the replication cycle. Unlike oncornaviruses where mitosis is essential for virus replication (4—7, 20) in the CELO/CEF system initiation of virus multiplication is dependent on events in the S phase of the cell replication cycle. The process of CELO virus multiplication in chicken embryo fibroblasts therefore seems to be more similar to the replication of parvoviruses H-1 (15, 16) and Lu III (17, 18), SV40 (14), polyomavirus (21, 22), and equine abortion herpes virus (9).

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